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14. ABSTRACT
The purpose of this research is to determine mechanisms through which hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), environmental degradation product of high energetic munition hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), causes persistent anemia in the rat. We have hypothesized MNX targets hematopoeitic stem cells and, like other myelosuppressive chemicals, will be fibrogenic to the bone marrow. Findings of this period are: 1) additional MNX suppressive effects on peripheral blood cells of myeloid lineage and similar effects of RDX and 2) suppression of bone marrow erythroid and myeloid stem cells of bone marrow by both MNX and RDX. Myeloid development appears more sensitive than erythroid, especially to RDX. These results suggest that MNX- and RDX toxicity in the rat appears to mimic some clinical manifestations of the myeloproliferative disorder, idiopathic myelofibrosis, and thus may offer a model for study of disease progression and intervention strategies. With respect to remediation of RDX-contaminated sites, collectively these data argue that risk of adverse hematological effects from exposure are lessened upon natural remediation to nitro reduced products.

15. SUBJECT TERMS
hexahydro-1,3,5-trinitro-1,3,5-triazine; hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine; RDX; MNX; anemia; myelosuppression

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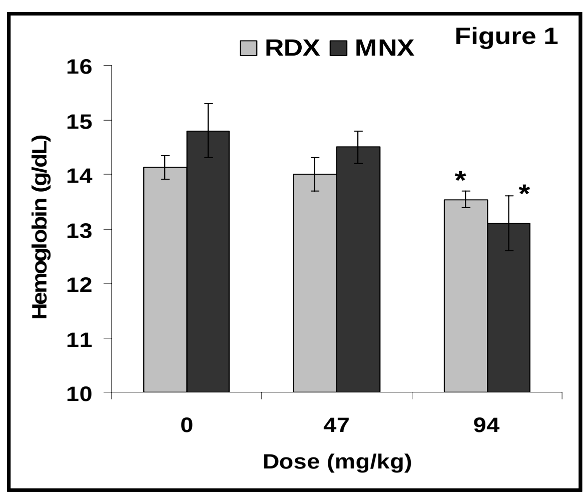
INTRODUCTION: The subject of research supported by this grant is a determination of the mechanism through which hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), environmental degradation product of high energetic munition hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), causes anemia. Anemia was detected in our previous acute toxicity studies in the rat (Meyer et al. 2005) and persisted 14 days after a single dose of MNX (NOAEL 47 mg/kg). Since anemia resulting from direct chemical destruction of intravascular erythrocytes is typically resolved within ~7 days in the rat, the 14-day persistence led us to hypothesize that MNX was cytotoxic to erythroid-lineage progenitor cells. Like other myelosuppressive chemicals, we also postulated that longer term, repeat exposure to MNX would be fibrogenic to the bone marrow microenvironment necessary for maturation of hematopoietic stem cells and hence offer an experimental model analogous to human idiopathic myelofibrosis. Further, previous studies on detection of a MNX ring cleavage product suggested this toxicity could be due to metabolism by bone marrow stromal cells. The scope of the proposed work encompasses determination of whether: 1) MNX produces persistent bone marrow toxicity, 2) MNX and ring cleavage metabolite MDNA, as produced from metabolism by bone marrow stromal cells, accumulate in bone after acute exposure; 3) acute exposure to MNX produces toxicity to bone marrow progenitors of the erythroid lineage; 4) acute exposure to MNX produces toxicity to the bone marrow stromal microenvironment, and 5) repeated administration of lower doses of MNX produces bone marrow toxicity including fibrosis. In addition, effects of parent RDX on selected endpoints will be assessed to provide structure-activity information relevant to mechanism of hematotoxicity and necessary for assessment of relative risk of the two nitramines in remediation of RDX-contaminated sites.

BODY:

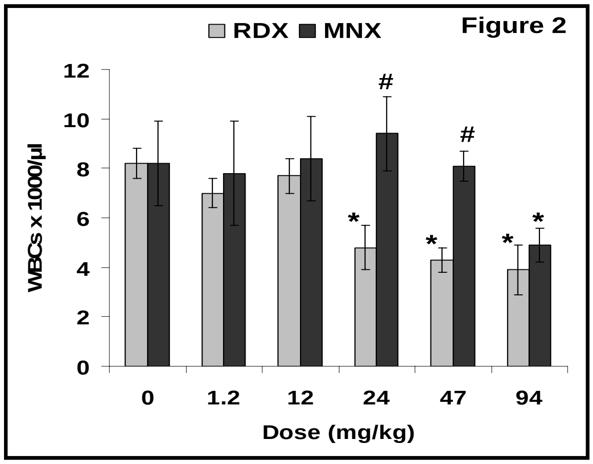
Task 1: Determination of whether acute exposure to MNX produces persistent bone marrow toxicity to include examination of selected endpoints in RDX-exposed rats (Months 1 – 12).

Accomplishments relevant to Task 1 include a comparative study of peripheral blood cell counts for MNX and parent RDX. Method: Female Sprague-Dawley rats (~200 g) were randomly assigned to treatment groups (n=5). Treatments were MNX and RDX at varying concentration up to 94 mg/kg (LD_{50} ~ 190 mg/kg) or vehicle (5% DMSO in corn oil) administered by oral gavage. Rats were observed for incidence of tremors and/or convulsions frequently over the first 8 hrs after treatment and euthanized if moribund. Survivors were weighed at 7 and 14 days, then exsanguinated by cardiac puncture while under CO_2 anesthesia. Blood was collected in heparinized syringes and transferred to EDTA-containing tubes for hemocytology. Hemoglobin and red and white blood cell count and size were determined with a CELL-DYN 3500 System (Abbott Laboratories; Abbott Park, IL). Hematocrit is derived from red blood cell size and count. Hemoglobin was measured as absorbance at 540 nm after leukocyte lysis and conversion to hemoglobin-hydroxylamine.

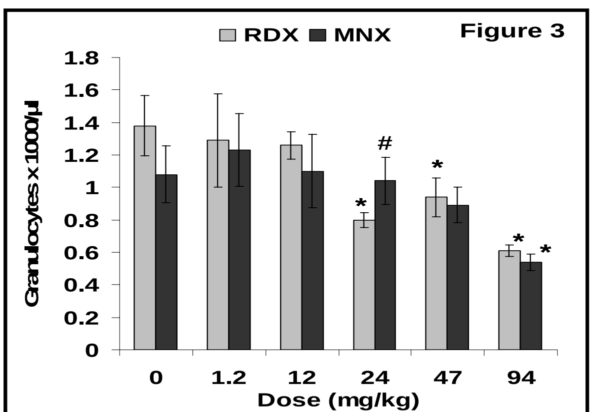


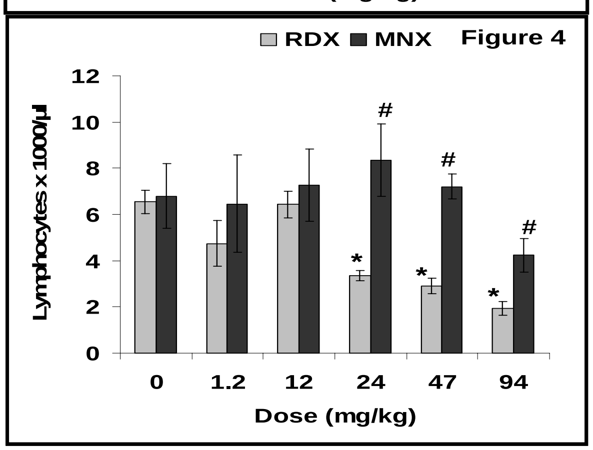
Results: As illustrated in Figure 1, we have determined that RDX, like MNX, causes an anemia that persists 14 days after a single dose. A statistically significant reduction in blood hemoglobin concentration was determined after treatment with both RDX and MNX at 94 mg/kg (*, $p<0.05$). Similar results were observed for hematocrit and mean corpuscular hemoglobin (MCH). The no-observed-adverse-effect-level (NOAEL) for both compounds was 47 mg/kg. Since rats receiving 94 mg/kg MNX lost weight during the 14 d post-exposure period (mean ± SEM = -9±6 g), the observed anemia could have resulted from iron deficiency. However, MNX effect on MCH indicated MNX-induced anemia was normochromic and thus unlike the hypochomic anemia of iron deficiency. High dose RDX-treated rats maintained body weight gain equivalent to controls (26±9 vs. 24±2 g for 94 vs 0 mg/kg).

Figure 2 summarizes the effects of RDX and MNX on blood leukocytes (WBCs). Differential effects were observed for MNX and RDX for this blood cell type; RDX was more potent in reducing leukocyte count. NOAEL for RDX was 12 mg/kg and for MNX 47 mg/kg (*indicates statistically significant difference from vehicle control and # indicates difference between MNX and RDX at the same dosage level, $p < 0.05$).



Blood leukocytes were further differentiated into granulocyte and lymphocyte populations based upon granularity and size. Figures 3 and 4 illustrate the effects of RDX and MNX on granulocytes and lymphocytes, respectively.



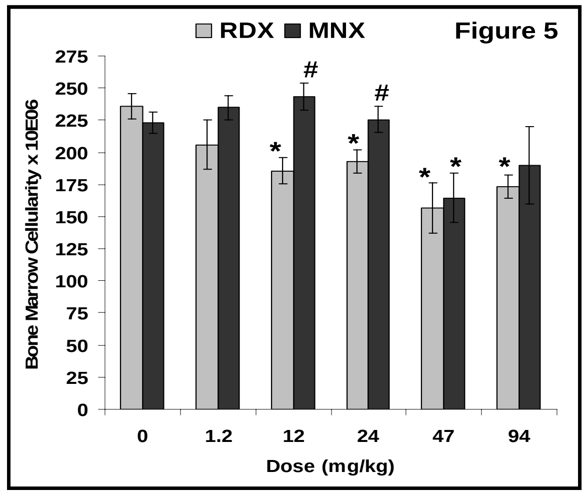


As observed with undifferentiated WBCs, both RDX and MNX decreased peripheral granulocytes and RDX was of higher potency than MNX. NOAELs for RDX and MNX were 12 and 47 mg/kg, respectively (*, # notation as in Fig. 2).

Peripheral granulocytes are comprised of neutrophils, basophils, eosinophils and monocytes. Although the CELL-DYNE blood analyzer distinguishes among these subpopulations, gates are set based upon human parameters. Thus, to assess which population(s) is affected by the nitamines in our rat studies, we are currently counting cell types manually on Wright-Giemsa-stained blood smears. This effort has been facilitated by upgrading our compound microscope to include a trinocular head, a small equipment purchase budgeted from this grant, that has enabled digital imaging Early data suggest that neutrophils are the granulocyte subpopulation most affected by both compounds.

Blood lymphocytes were clearly affected differentially by RDX and MNX. As shown in Figure 4, only RDX at doses greater than 12 mg/kg significantly decreased blood lymphocyte numbers

We directly monitored bone marrow cellularity as affected by these nitramines to determine whether bone marrow toxicity and consequential depletion of hematopoietic stem cells could account for the loss of peripheral erythrocytes and leukocytes observed with RDX and MNX. Method: Femurs from each rat were disarticulated from the pelvis and the proximal and distal heads of each cutoff with a razor blade. Bones were flushed with 3 ml ice-cold Iscoves Modified Dulbecco Medium (IMDM) plus 2 % FBS and antibiotic/antimycotic. Residual fluid is flushed from the bones with a 3 ml bolus of air and bones were then flushed a second time with IMDM medium. Flushes from each animal were pooled and filtered through nylon mesh and centrifuged (200 x g, 10 min). Pelleted cells were resuspended in IMDM medium and total cell number counted with a hemocytometer.



Results: Both the chemicals were found to decrease the bone marrow cellularity (Figure 5). Similar to results with leukocyte cellularity of peripheral blood, RDX was more potent than MNX. NOAELs for RDX and MNX were 1.2 and 24 mg/kg, respectively (*indicates statistically significant difference from vehicle control and # indicates difference between MNX and RDX at the same dosage level, $p < 0.05$).

To summarize Task #1 accomplishments, we have provided evidence supporting the hypothesis that MNX-induced, persistent anemia results from toxic effects of this chemical on hematopoietic stem cells of the bone marrow. Further, we have obtained data suggesting that not only the erythroid lineage is affected, but that MNX toxicity to bone marrow has consequences on the myeloid lineage as well. Importantly, we have determined that the parent chemical RDX of environmentally produced MNX has equivalent effects on the erythroid linege, but is of greater potency for adverse effects on cells of the myeloid lineage. RDX, but not MNX, hematotoxicity extends to the lymphocytic lineage.

Task 2: Determination of whether MNX and metabolite MDNA accumulate in bone after acute exposure and whether bone marrow stromal cells are metabolically capable of converting MNX to MDNA. (Months 13 – 36).

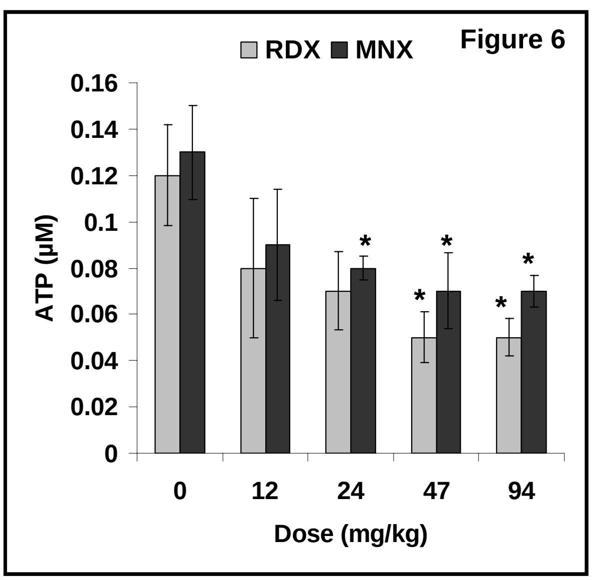
A manuscript describing previous collaborative work with U.S. Army Corps of Engineers colleagues (MacMillan, Denise, et al.) on disposition and metabolism of RDX and MNX is currently undergoing internal review and will be submitted for journal publication shortly. The Meyer lab contribution to this project was funded through other DoD support (DACA42-02-P-0035). However, one observation from that project especially salient to the present report is that of detection of RDX and ring cleavage product MDNA in tissues of MNX-exposed rats. This further justifies future pursuit of Task #2 activities.

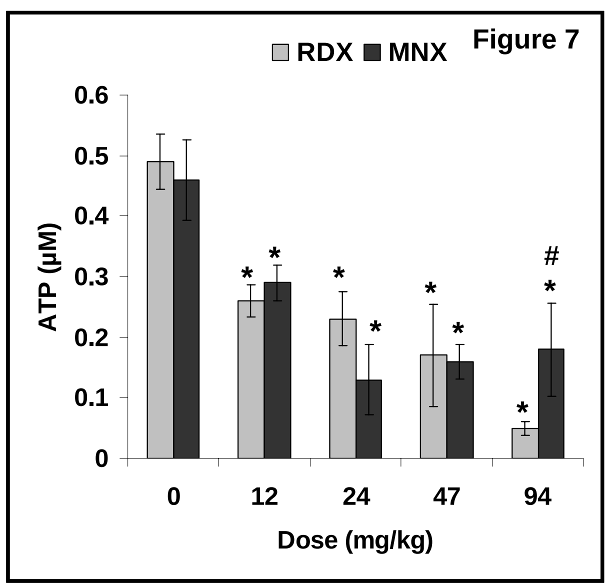
A recently admitted graduate student, Mitchell Wilbanks, started his program August 2006. Mr. Wilbank's thesis project will be to accomplish the activities of Task #2.

Task 3: Determination of whether acute exposure to MNX produces toxicity to bone marrow progenitors of the erythroid and myeloid lineages. (Months 18 – 36)

Accomplishments relevant to Task 3 include a comparative study of effects of RDX and MNX on hematopoietic stem cells of the bone marrow. Method: Total bone marrow cells were isolated from femurs as described above. Mononuclear cells were isolated using Histopaque-1077™ (Sigma Chemical Co, USA) according to supplier's protocol. Separated mononuclear cells were counted using a hemocytometer. Progenitor cells of the myeloid (CFU-GM) and erythroid (BFU-E) lineages were assayed using Halo kits as per supplier's instruction (Hemogenix Inc, USA). In brief, 20,000 mononuclear cells were treated with growth factors, methyl cellulose, serum and incubated in 96-well plates in a CO_2 incubator for 5 days. After 5 days, colony forming activity was monitored in a high through-put system in which cell numbers were deduced from fluorescent measurements of amount of ATP per well. Amount of ATP was quantified against the standard curve generated on the same day.

Results: Figure 6 and 7 illustrate the effects of RDX and MNX on colony forming ability of erythroid and myeloid progenitor cells. Both RDX and MNX were found to dramatically decrease Burst Forming Units-Erythroblasts (BFU-E) and Granulocyte/Macrophage-Colony Stimulating Forming Units (CFU-GM). Both nitramines were effective at relatively low doses with the myeloid CFU-GM (Figure 7) being more susceptible to RDX and MNX suppression. NOAEL for RDX and MNX were 24 and 12 mg/kg, respectively, on erythroid BFU-E cells (Figure 6). For CFU-GMs, NOAELs was not identified since all doses tested (12, 24, 47 and 94 mg/kg) for both compounds showed significant lower colony formation ability than that of vehicle controls. RDX was more effective than MNX at 94 mg/kg dose in suppressing CFU-GM cells (Figure 7; *, # notation as in Fig. 5).





Flow cytometry: We have also proposed to assess the relative developmental state of the erythroid lineage cells targeted by the nitramines. These studies utilize flow cytometric assessment of frequency of cells expressing early (Thy1.1) and late (CD71; transferrin receptor) erythroid lineage markers. Senior Ph.D. candidate, Vijay Kale, completed a 2-week training session conducted by BD Biosciences in Spring 2006 on the use of the FACSCalibur flow cytometer, a shared resource of the ULM College of Pharmacy. Mr. Kale is currently optimizing conditions for his flow cytometric studies on Thy1.1- and CD71-labeled rat bone marrow cells.

Accomplishments under Task #3 have directly addressed RDX and MNX effects on bone marrow progenitor cells and studies have provided data consistent with the hypothesis that these compounds are targeting hematopoietic stem cell development in the bone marrow. Although unexpected based upon our earlier studies focused upon the erythroid lineage, these newer studies unequivocally demonstrate comparable or greater sensitivity of myeloid precursor cells. The coordinate suppression of both myeloid and erythroid lineages further suggest that a more upstream precursor cell common to both lineages may be affected or, alternately, the supportive environment of the stem cell niche may be compromised. The latter will be addressed in future Task #4 and #5 studies.

<u>Task 4</u>: Determination of whether acute exposure to MNX produces toxicity to the bone marrow stromal microenvironment. (mos. 12 – 30)
<u>Task 5</u>: Determination of whether repeated administration of lower doses of MNX produces bone marrow toxicity, especially fibrosis. (mos. 24 – 36).

Mr. Wilbank's future studies (Task #2) will concurrently address stromal colony forming units. Additionally, graduate student Melissa Aycock, who started in the Meyer lab January 2006, will contribute to this Tasks 4 and 5. Ms. Aycock is currently conducting pilot studies to optimize histopathological techniques for bone in preparation of conducting repeat dose studies to detect bone marrow fibrosis. Determination of fibrogenic growth factors by immunoassay will be done in the same or parallel studies.

<u>Statistics:</u> Effects of MNX and RDX on hematological parameters were determined by ANOVA with post-hoc comparisons of treatment means against vehicle control done with Dunnett's test and difference between MNX and RDX was assessed with a student's *t*-test. Results were considered statistically significant with $p < 0.05$. Data were statistically analyzed using JMP 4.0.4 software (SAS Institute Inc.).

KEY RESEARCH ACCOMPLISHMENTS:

Studies thus far have demonstrated:

- support for the hypothesis that MNX-induced, persistent anemia results from toxic effects of this chemical on hematopoietic stem cells of the bone marrow.
- suggested that not only the erythroid lineage is affected, but that MNX hematotoxicity has consequences on the myeloid lineage as well, thus implicating a common up-stream multipotential stem cell and/or the stromal microenvironment as a target.
- determined that the parent chemical RDX of environmentally produced MNX has equivalent effects on the erythroid linege, but is of greater potency for adverse effects on cells of the myeloid lineage and unique effects on the lymphocytic lineage.

REPORTABLE OUTCOMES:

1. Poster and platform presentations at 2006 Military Health Research Forum, San Juan, Puerto Rico, May 1-4, 2006.

Meyer, SA Persistant anemia associated with hematotoxicity of nitro reduced degradation product MNX of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX).

2. Abstract accepted for presentation at 2007 annual meeting of the Society of Toxicology, Charlotte, NC

Kale, V.M., M.M. Aycock, M.S. Wilbanks, E.J. Perkins, L.S. Inouye. Hematotoxicity of munitions compound hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and environmental degradation product MNX. *The Toxicologist. 2007; 91 (S-1): in press.*

<u>CONCLUSION</u>: Results of these studies thus far support the hypothesis that hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), environmental degradation product of high energetic munition hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), is toxic to hematopoietic bone marrow stem cells. Data suggest toxicity to an early erythroid/myeloid lineage precursor and/or to the bone marrow stromal niche supporting hematopoiesis. Thus, previously observed peripheral blood disorders in MNX-treated rats appear to be the consequence, in part, of loss replenishing stem cell populations. As such, this system appears to mimic some clinical manifestations of the myeloproliferative disorder, idiopathic myelofibrosis, and thus offer a model for study of possible mechanisms of disease progression and development of intervention strategies.

Additionally, we are able to conclude that the parent RDX is more potent than MNX in myelosuppression. In addition to the adverse hematological effects we have documented, these results suggest additional functional consequences with respect to host resistance to infection, inflammation and tissue trauma. As relates to the role of the Department of Defense in remediation of RDX-contaminated sites, **collectively these data argue that risk of adverse hematological effects from exposure are lessened upon natural remediation to nitro reduced products**.

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Meyer, S.A., A.J. Marchand, J.L. Hight, G.H. Roberts, L.S. Inouye, L.B. Escalon and D.K. MacMillan. Up-and-down procedure (UDP) determination of acute toxicity of nitroso degradation products of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). J. Appl. Toxicol. 25:427-434, 2005

APPENDICES: None

SUPPORTING DATA: None